

Activity of mannose-binding lectin in centenarians

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Summary

We analyzed MBL2 gene variants in two cohorts of centenarians, octo-nagenarians and nonagenarians, and in the general population, one from Sardinia Island (Italy), recruited in the frame of the AKea study, and another from Campania (southern Italy), to search for haplotypes related to longevity. We also assessed *in vitro* the effect of mannose-binding lectin (MBL) on various human cells at different stage of senescence. The frequency of high and null activity haplotypes was significantly lower, and the frequency of intermediate activity haplotype significantly higher in centenarians and in subjects between 80 and 99 years from both the cohorts as compared each to the general population from the same geographic area. Furthermore, serum MBL concentration (also after normalization to serum albumin) was significantly lower in centenarians and in octo- and nonagenarians as compared to the general population, suggesting that intermediate MBL haplotype/activity may be protective. We also demonstrated that *in vitro* MBL protein bound to senescent IMR90 fibroblasts thereby causing cell lysis, but not to other types of cycle-arrested cells not in senescence. This implicates a novel role of MBL in the clearance of senescent cells.

Key words: aging; haplotypes; innate immunity; mannose-binding lectin; senescence.

Introduction

Mannose-binding lectin (MBL) is an acute-phase protein produced by the liver and encoded by the MBL2 gene. In liver cells, the protein forms triple helices that once released in serum polymerize thereby forming dimers to octamers (Lipscombe *et al.*, 1995), and the biologic activity of MBL

depends on the degree of serum polymerization (Dommett *et al.*, 2006). The protein interacts with surface sugars of specific microorganisms and plays a role in innate immunity by functioning as a pattern-recognition molecule. Indeed, it: (i) activates the lectin-complement pathway; (ii) promotes opsonophagocytosis; and (iii) modulates inflammation (Dommett *et al.*, 2006).

Three variants in noncoding regions of MBL2 have been identified: H/L (nucleotide –550), X/Y (nucleotide –221), and P/Q (at the +4 in the 5' UTR). These variants reduce synthesis and serum levels of the protein. Three other variants involve codons 52, 54, and 57 and are called D, B, and C alleles, respectively. They impair serum polymerization and thus reduce the biologic activity of MBL (Garred *et al.*, 2006). The six MBL2 variants are in linkage disequilibrium, and their combination results in seven common haplotypes that correspond to high (HYPA and LYQA haplotypes), intermediate (LYPA haplotype), low (LXPA haplotype), and null (HYPD, LYPB, LYQC haplotypes) biologic activity of the protein (Verdu *et al.*, 2006; Boldt *et al.*, 2010).

Subjects bearing low and null haplotypes experience severe infections particularly in early childhood before the onset of specific protection by adaptive immunity (Dommett *et al.*, 2006). Moreover, MBL null haplotypes are a negative modifier factors in cystic fibrosis because they cause a more severe liver (Tomauiuolo *et al.*, 2009) and pulmonary (Garred *et al.*, 1999) disease. In addition, the HYPD-null haplotype is a risk factor for gastric cancer in subjects bearing *Helicobacter pylori* (Scudiero *et al.*, 2006). Finally, null MBL haplotypes are associated with more severe autoimmune diseases (Maury *et al.*, 2007; Gergely *et al.*, 2009; Jacobsen *et al.*, 2009).

Mannose-binding lectin 2 variants are frequent worldwide (Dommett *et al.*, 2006; Garred, 2008). For example, we identified MBL variant haplotypes corresponding to intermediate, low, or null activity in about 50% of 550 subjects from the general population of southern Italy (Scudiero *et al.*, 2006). It has been postulated that MBL plays a redundant role in human immunity and that MBL2 variants do not affect population fitness, and thus, their frequency can increase (Verdu *et al.*, 2006). The high heterozygosity of MBL2 may be due to a series of advantages coming from the lower activity of the protein, namely, a less severe expression of rheumatoid arthritis (Garred *et al.*, 2000; Jacobsen *et al.*, 2009), Sjögren disease (Ramos-Casals *et al.*, 2009), vasculitis (Xing *et al.*, 2009), and Crohn's disease (Velavan *et al.*, 2010), whereas high MBL activity causes more severe infections by intracellular pathogens that use C3 receptor to enter the host (Dommett *et al.*, 2006), e.g., visceral leishmaniasis (Peres *et al.*, 2007) and malaria (Boldt *et al.*, 2006).

We used two approaches to evaluate the effect exerted by MBL variants: (i) we analyzed MBL2 variants in centenarians, octo-nagenarians, and control groups from two different Italian regions to search for MBL2 haplotypes related to longevity; and (ii) we assessed the effect of MBL on several human cell models at different senescence stages.

Results

Mannose-binding lectin haplotypes

Table 1a shows the results obtained in Sardinian population. The frequency of high activity MBL haplotypes was significantly lower in centenarians than in the general population (23.2% vs. 42.9%; $P < 0.001$). The frequency of these haplotypes was also significantly lower ($P < 0.01$) in octo-nagenarians than in the general population. Furthermore, null

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Table 1 Allele frequency *n* and (%) of mannose-binding lectin (MBL2) haplotypes in subjects of different age groups from Sardinia (a) and from Campania (b)

MBL activity		High			Intermediate	Low	Null			
Groups	No. of alleles	HYPA	LYQA	All high (HYPA + LYQA)	LYPA	LXPA	HYPD	LYPB	LYQC	All null (HYPD + LYPB + LYQC)
(a)										
> 100 years	142	22 (15.5)	11 (7.8)**	33 (23.2)**	55 (38.7)**	32 (22.5)	1 (0.7)	20 (14.1)	1 (0.7)	22 (15.5)*
80–99 years	300	68 (22.7)	28 (9.3)**	96 (32.0)*	76 (25.3)**	48 (16.0)	10 (3.3)	53 (17.7)	17 (5.7)	80 (26.7)
General population	366	88 (24.0)	69 (18.9)	157 (42.9)	42 (11.5)	69 (18.9)	14 (3.8)	69 (18.9)	15 (4.1)	98 (26.8)
(b)										
> 100 years	118	32 (27.1)	10 (8.5)*	42 (35.6)**	32 (27.1)**	35 (29.7)	1 (0.9)	7 (5.9)	1 (0.9)	9 (7.6)*
80–99 years	252	71 (28.2)	28 (11.1)*	96 (38.1)**	46 (18.3)**	64 (25.4)	8 (3.2)	30 (11.9)	3 (1.2)	47 (18.7)
General population	1106	390 (35.3)	198 (17.9)	588 (53.2)	52 (4.7)	255 (23.1)	49 (4.4)	144 (13.0)	18 (1.6)	212 (19.0)

Significantly different *($P < 0.01$) and **($P < 0.001$) vs. the general population.

haplotypes were significantly less frequent in centenarians than in the general population ($P = 0.01$). Finally, the intermediate activity haplotype (LYPA) was significantly more frequent in centenarians than in the general population (38.7% vs. 11.5%; $P < 0.001$). This haplotype was also significantly more frequent in octo-nonenarians than in the general population (25.3% vs. 11.5%; $P < 0.001$). Table 1b reports the data obtained from the population of Campania. The results replicated those obtained in Sardinian population. In fact, the frequency of the high activity MBL haplotypes was significantly lower either in centenarians (35.6%, $P < 0.001$) or in octo-nonenarians (38.1%, $P < 0.001$), each compared to the general population (53.2%). Furthermore, the frequency of null activity haplotypes obtained in centenarians (7.6%) was significantly lower as compared to that obtained in the general population (19.0%, $P = 0.01$). Finally, the frequency of the intermediate activity haplotype (LYPA) was significantly higher in either centenarians (27.1, $P < 0.001$) or octo-nonenarians (18.3, $P < 0.001$) each compared to the general population (4.7%).

Table 2 reports the genotypes identified in the three groups of subjects. Although statistical analysis was precluded because of the low number of cases, a lower percent of centenarians was homozygous for high (four cases from Sardinia and seven from Campania) or homozygous for null haplotypes (one case from Sardinia and none from Campania) compared with the general population.

Serum mannose-binding lectin

Either in the population from Sardinia and in that from Campania, the values of serum MBL, albumin, and the MBL/albumin ratio were significantly lower either in centenarians or in octo-nonenarians (Table 3) as compared to the general population from the same geographic area.

Mannose-binding lectin activity toward cells

To define the number of senescent cells, we measured beta-galactosidase activity of IMR-90, NHBE cells, and primary nasal epithelial cells at increasing population doubling (from PD 39, young, to 54, senescent, for IMR-90 cells; from PD 7 to 15 for NHBE cells; and from day 7 to 25 for primary nasal epithelial cells). To quantify cells positive to beta-galactosidase staining, we randomly selected for each group of cells at different PD five areas of observation and calculated the mean number. The percentage of IMR-90-stained cells significantly ($P < 0.001$) increased from early passages (PD 39) to late ones (PD 54, see Table 4a and Fig. 1a). No NHBE cells or primary epithelial cells were positive (Table 4b,c and Fig. 1b,c), at any doubling stage.

Table 2 Number and frequency of mannose-binding lectin (MBL2) genotypes subjects of different age groups from Sardinia (a) and from Campania (b)

	High	Intermediate	Low	Null
(a)				
> 100 years ($n = 71$)				
High	4 (5.6)			
Intermediate	13 (18.3)	13 (18.3)		
Low	6 (8.5)	10 (14.1)	4 (5.6)	
Null	6 (8.5)	6 (8.5)	8 (11.3)	1 (1.4)
80–99 years ($n = 150$)				
High	18 (12.0)			
Intermediate	15 (10.0)	18 (12.0)		
Low	17 (11.3)	7 (4.7)	4 (2.7)	
Null	28 (18.7)	18 (12.0)	16 (10.7)	9 (6.0)
General population ($n = 183$)				
High	37 (20.2)			
Intermediate	8 (4.4)	11 (6.0)		
Low	27 (14.8)	9 (4.9)	7 (3.9)	
Null	40 (21.9)	11 (6.0)	19 (10.4)	14 (7.7)
(b)				
> 100 years ($n = 59$)				
High	7 (11.9)			
Intermediate	14 (23.7)	4 (6.8)		
Low	13 (22.0)	8 (13.6)	4 (6.8)	
Null	1 (1.7)	2 (3.4)	6 (10.2)	0
80–99 years ($n = 126$)				
High	28 (22.2)			
Intermediate	10 (7.9)	8 (6.4)		
Low	23 (17.8)	11 (8.7)	7 (5.6)	
Null	10 (7.9)	9 (7.1)	18 (14.3)	2 (1.6)
General population ($n = 553$)				
High	153 (27.7)			
Intermediate	21 (3.8)	2 (0.4)		
Low	149 (26.9)	12 (2.2)	32 (5.8)	
Null	112 (20.3)	15 (2.7)	30 (5.4)	27 (4.9)

To assess the MBL binding to senescent cells, we set up an immunofluorescence assay using cells of different ages incubated with human MBL protein followed by anti-human MBL mAb and FITC-conjugated anti-mouse IgG. Each experiment was conducted five times, and the results are the mean of the five experiments. As shown in Table 4a, IMR-90 cells at PD 39 were negative to staining. At PD 46, 3.6% cells were fluorescent and staining significantly ($P < 0.01$) increased to 10.3% of cells at 54 PD (Table 4a).

Table 3 Serum mannose-binding lectin (MBL), albumin, and MBL/albumin ratio, mean and SD in the three groups of subjects under study from Sardinia (a) and from Campania (b)

Groups	N	Serum MBL (ng mL ⁻¹)	Serum albumin (mm)	MBL/albumin ratio
(a)				
> 100 years	71	879.6 (388.9)**	37.0 (2.4)**	23.7 (7.3)**
80–99 years	150	1126.5 (529.0)**	38.2 (2.9)**	33.0 (6.4)**
General population	80	1365.4 (568.1)	41.2 (3.3)	37.4 (14.4)
(b)				
> 100 years	59	1001.8 (393.7)**	37.0 (2.5)**	27.1 (10.6)**
80–99 years	126	939.3 (461.5)**	38.1 (3.0)**	24.3 (12.3)**
General population	160	1300.4 (623.8)	40.3 (3.0)	32.6 (15.6)

Significantly different *($P < 0.01$), **($P < 0.001$) as compared to the general population.

Table 4 Beta-galactosidase and mannose-binding lectin (MBL) binding assay of IMR-90, NHBE, and primary nasal epithelial cells at different ages

PD cells	% Beta gal cells (mean of five assays)		% MBL-stained cells (mean of five assays)	
(a) IMR 90				
39	0		0	
46	34.6 (± 12.1)	$P < 0.001$	3.6 (± 1.3)	$P < 0.001$
54	82.6 (± 14.0)		10.3 (± 4.8)	
(b) NHBE				
PD cells	% Beta gal cells (mean of three assays)		% MBL-stained cells (mean of five assays)	
7	0		0	
10	0		0	
15	0		0	
(c) Primary nasal epithelial cells				
Days in culture	% Beta gal cells (mean of three assays)		% MBL-stained cells (mean of five assays)	
7	0		0	
14	0		0	
25	0		0	

To verify that staining was because of MBL binding (and thus Ca⁺⁺ dependent), we repeated the experiment: (i) adding 10 mM EDTA with no calcium and (ii) adding mannose. Both EDTA and mannose blocked the binding of MBL protein to senescent cells. Indeed, no cell was positive to FITC antibody (data not shown).

The same immunofluorescence assay was carried out for NHBE and primary nasal epithelial cells even if they were negative to beta-galactosidase staining. As expected, no fluorescent signal was detected in these cells at any age (Table 4b,c). Finally, we assessed lactate dehydrogenase (LD) values in the culture medium of IMR-90 cells before and after incubation with MBL. All experiments were performed five times, and the data reported in Table 5 are the mean of five assays. The addition of MBL caused a significant ($P < 0.01$) increase in LD in the culture medium (from 74 to 120 U L⁻¹). There was a further (not statistically significant) increase after the addition of fresh human serum (120–144 U L⁻¹ after the subtraction of the LD value obtained in serum alone). The same experiments were performed on medium of NHBE and primary nasal epithelial cells, and the addition of MBL and MBL plus serum did not affect LD values in the medium (data not shown).

Finally, we looked for mannosyl residues on young and senescent IMR-90 cells (PD 39 and PD 54) using the staining for concanavalin A, a lectin that recognizes specific sugar residues, in particular mannosyl residues. Old IMR-90 cells (PD 54) bound about 40% more concanavalin A than the youngest ones (PD 39).

Discussion

Our study revealed a peculiar MBL2 haplotype associated with longevity. In fact, in centenarians, we observed: (i) as expected, a low frequency of haplotypes associated with null protein activity (i.e., LYPB, LYQC, and HYPD) and only one case of a homozygous genotype for null haplotypes; (ii) a surprisingly low frequency of haplotypes associated with high protein activity (i.e., HYPA and LYQA) and only a few cases homozygotes for high activity haplotypes; (iii) a high frequency of the LYPA haplotype, which is associated with intermediate MBL activity and a high percentage of compound genotypes for this haplotype. Furthermore, MBL serum concentration was significantly lower in centenarians than in the general population, even after normalization to serum albumin (which neutralizes the effect of the reduced liver prothyrosynthesis due to age). A similar trend was observed in octo-nonenarians vs. the general population.

Such MBL2 haplotypes may confer some advantages and thus may contribute to healthy aging. In fact, MBL2-null haplotypes (that are very rare in our centenarians) are related to a wide spectrum of infections particularly in early childhood (Dommett *et al.*, 2006) and to noninfectious diseases in adults (Scudiero *et al.*, 2006; Maury *et al.*, 2007; Jacobsen *et al.*, 2009). Therefore, it is not surprising that these haplotypes are rarely found in centenarians. It is more difficult to explain the low allelic frequency of haplotypes with high activity observed in our long-lived individuals. Various disadvantages have been associated with high MBL activity. For example, a high frequency of MBL variants was found in subjects from southern Africa (Lipscombe *et al.*, 1992) and southern America (Madsen *et al.*, 1998), and the resulting low MBL activity has been related to less severe malaria (Boldt *et al.*, 2006) and to a lower frequency and severity of leishmaniasis (Peres *et al.*, 2007). But unlike southern Africa and southern America, Sardinia and Campania are not areas under a high parasitic burden. Indeed, the occurrence of antibodies against such parasites in our subjects is comparable to that of other European areas (data not shown). A second disadvantage of a high MBL activity is the excessive complement activation that results in immunopathologically mediated host damage (Lipscombe *et al.*, 1992), which may lead to more severe forms of autoimmune diseases (Maury *et al.*, 2007; Gergely *et al.*, 2009; Jacobsen *et al.*, 2009). In this context, it is noteworthy that none of our centenarians has clinically evident autoimmune diseases and a review of their clinical record excludes previous relevant autoimmune disorders.

In addition, we demonstrate that MBL binds the surface of senescent fibroblasts, a cell system was used for a long time to study cell senescence (Hayflick, 1980; Killilea & Ames, 2008). The addition of human serum increased the percentage of cells that were bound by MBL. This may reflect the fact that MBL binds MASPs in serum, and this interaction increases the affinity for the target (Garred *et al.*, 2006), as described in neoplastic cells (Ma *et al.*, 1999). The binding of MBL to senescent fibroblasts caused lysis, as indicated both by MBL fluorescence in the cytoplasm and by the increased release of LD in the medium. The interaction of MBL with membrane is peculiar to senescent cells; in fact, MBL binds only IMR-90 cells, at the end of PD, that express high levels of beta-galactosidase, a well-known marker of cellular senescence (Dimri *et al.*, 1995). On the contrary, MBL does not interact with NHBE cells and primary nasal epithelial cells even at the end of population doubling, because these cells do not express the senescent phenotype (no expression of beta-galactosidase).

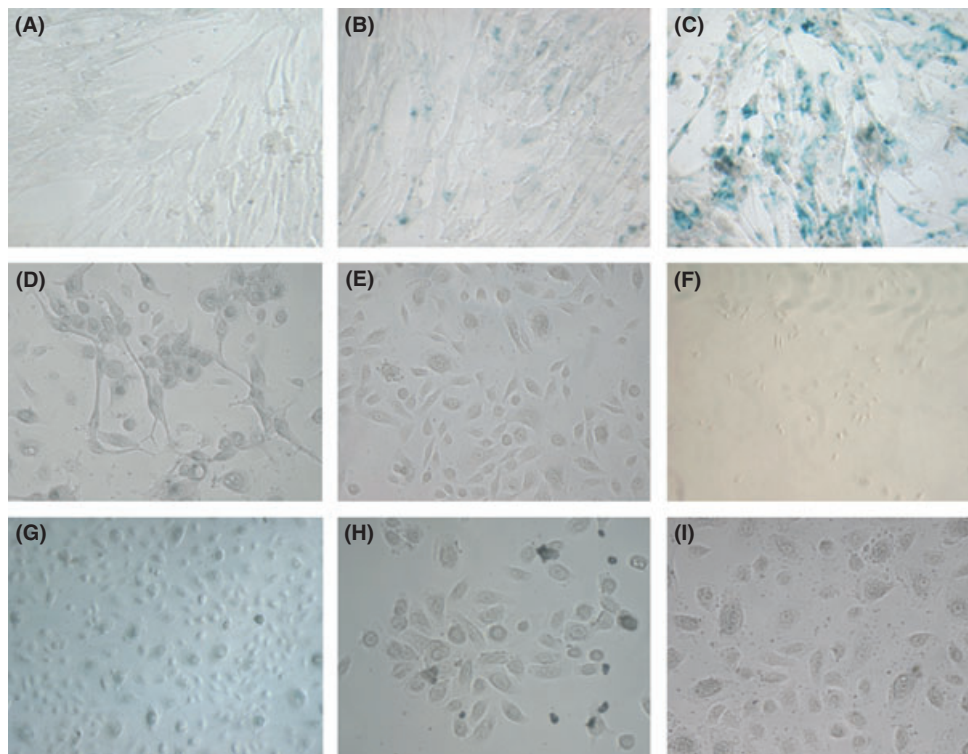


Fig. 1 Beta-galactosidase staining ($\times 20$) of IMR-90 (a–c), NHBE (d–f), and primary human epithelial cells (g–i) at different ages. IMR-90 cells at early passage culture (PD 39) (a), at middle-passaged culture (PD 46) (b), and at late-passaged culture (PD 54) (c) undergo growth arrest and extensively express beta-galactosidase. NHBE cells at early passage culture (PD 7) (d), at middle-passaged culture (PD 10) (e), and at late-passaged culture (PD 15) (f). Primary nasal epithelial cells at early passage culture 7 days (g), at middle-passaged culture 14 days (h), and at late-passaged culture 25 days (i). No beta-galactosidase activity is present in neither NHBE nor nasal primary epithelial cells that express beta-galactosidase although at late passages both cell types undergo growth arrest.

Table 5 Lactate dehydrogenase (LD) activity in culture medium of IMR-90 cells before and after the addition of mannose-binding lectin (MBL). The results are the mean of five experiments

Time	LD activity (U L^{-1})	
0	74 (± 12.4)	$P < 0.01$
After MBL addition	120 (± 20.9)	
After serum addition	144 (± 27.7)	n.s.

and thus are quiescent or terminally differentiated cells (Dimri *et al.*, 1995). We speculate that also *in vivo* MBL might interact with senescent cells thereby contributing to their lysis, and consequently, an excessively high activity of MBL may result in stronger activation of the complement that promotes the lysis of senescent cells. Reduced activity of serum MBL (as observed in our long-lived subjects that bear the intermediate MBL2 haplotype and lower serum levels of the protein) may be associated with a less intense activity of MBL toward senescent cells, with the advantage of reduced autoimmune triggering in such subjects. This hypothesis goes to add to the relevant literature that reports that less active inflammatory genotypes are an advantage for longevity (Licastro *et al.*, 2005).

The activity of MBL toward senescent cells may depend on the remodulation of the surface sugar pattern observed in senescent cells (Blondal *et al.*, 1985), and these changes trigger the binding to senescent fibroblast of concanavalin A (as observed also in the present study), a plant lectin

that shows a high affinity for the same carbohydrate moieties, mainly mannose (Rüdiger & Gabius, 2001). Our data suggest that these changes may render a percentage of senescent fibroblasts also the target of MBL binding, and a similar situation could involve various types of human senescent cells. On the other hand, it is known that MBL binds some types of human cells. In particular, it is involved in late apoptosis binding apoptotic cells that express altered patterns of membrane carbohydrates (Rüdiger & Gabius, 2001; Nauta *et al.*, 2003); furthermore, changes in the surface sugar distribution during neoplastic transformation promote MBL binding to cancer cells (Roos *et al.*, 2004; Garred *et al.*, 2006), and the protein mediates cytotoxic effects (Blondal *et al.*, 1985; Hakomori, 2001).

To conclude, centenarians from Sardinia and Campania tell us that the most advantageous MBL2 haplotype for successful aging is the intermediate haplotype, which corresponds to intermediate levels of MBL serum activity. Furthermore, our data show that MBL is active – at least *in vitro* – toward senescent cells.

Materials and methods

Subjects

We studied three groups of subjects from Sardinia (Italy), unrelated up to the third generation: (i) 71 centenarians (27 men, mean age 100.9 ± 1.3 years); (ii) 150 octo-nagenarians, i.e., subjects between 80 and 99 years (52 men, mean age: 89.1 ± 6.0 years); and (iii) 183 subjects from the general population (94 men) whose sex and age distribution

match the anagraphic distribution of Sardinia. We have a complete computerized clinical record for each subject, within the frame of the AKea study (Deiana *et al.*, 1999; Poulain *et al.*, 2004) chaired by Prof. L. Deiana and approved by the Ethic committee of University of Sassari, Italy. Furthermore, we studied three groups of subjects from Campania (southern Italy) unrelated up to the third generation: (i) 59 centenarians (30 men, mean age: 101.6 ± 1.7); (ii) 126 octo-nagenarians, i.e., subjects between 80 and 99 years (59 men, mean age: 91.0 ± 4.7 years); and (iii) 553 subjects from the general population previously described (Scudiero *et al.*, 2006). All long-lived subjects are free from major diseases (i.e., cardiovascular, neoplastic, autoimmune, or neurodegenerative diseases). All patients are mobile; they practice regular activity as suggested by their physicians. Each subject provided the informed consent to be included in the study and written permission to use a biologic sample for anonymous biologic research. A DNA sample (extracted from peripheral blood with a commercial procedure) and a serum sample (stored at +4 and 80 °C, respectively, up to use) were available for each subject included in the study.

Mannose-binding lectin gene variants

We analyzed the six MBL2 gene variants (Garred *et al.*, 2006), using a PCR-reverse dot-blot procedure (Innolipa MBL2; Innogenetics, Ghent, Belgium). In line with current nomenclature, the three polymorphisms in the 5' noncoding regions of the gene are designated H/L (nucleotide -550), X/Y (nucleotide -221), and P/Q (at the +4 in the 5' UTR); the three MBL2 variants at codons 52, 54, and 57 are designated D, B, and C alleles, respectively. The wild-type allele at the three codons is defined allele A. Seven MBL2 haplotypes result from the combination of the seven variants and are named HYPA, LYQA (high activity haplotypes), LYPA (intermediate activity haplotype), LXPA (low activity haplotype), and HYPD, LYPB, and LYQC (null activity haplotypes, Verdu *et al.*, 2006; Boldt *et al.*, 2010). The MBL haplotype distribution in control subjects in Hardy-Weinberg equilibrium (Scudiero *et al.*, 2006). Furthermore, it is not influenced by sex (R. Tomaiuolo, unpublished results). So we analyzed together male and female subjects of each subgroup.

Serum MBL and albumin

Serum MBL was analyzed using a commercial ELISA procedure (Sanquin, Amsterdam, the Netherlands); serum albumin was analyzed by nephelometry (Roche, Milan, Italy). The data were expressed also as MBL/albumin ratio.

Cell culture

IMR-90 human embryonic lung fibroblasts were purchased from ATCC-LGC Standards (CCL-186; Middlesex, UK). Culture medium components were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA) or Lonza Inc. (Walkersville, MD, USA). IMR-90 fibroblasts were cultured at 37 °C with 5% CO₂ in Eagle's minimal essential medium containing 10% heat-inactivated fetal bovine serum supplemented with 1% penicillin/streptomycin solution (Sigma) and 2 mM L-glutamine. Cells were harvested by 0.5% (w/v) trypsin-EDTA solution (Gibco, Invitrogen, Carlsbad, CA, USA). All the experiments involved cultures from the population doubling (PD) 39–54. Normal human bronchial epithelial cells (NHBE) were obtained from Clonetics (Walkersville, MD, USA). Cells were cultured at 37 °C in humidified 5% CO₂ and grown in bronchial epithelial growth medium (Lonza Inc.) that consists of bronchial epithelial basal medium supplemented with rhEGF, bovine pituitary extract, insulin, hydrocorti-

sone, transferrin, retinoic acid, epinephrine, triiodothyronine, and gentamicin sulfate/amphotericin-B (GA-1000) (purchased from Lonza Inc.). NHBE cells were harvested and split according to the NHBE technical sheet and using Lonza ReagentPack (Lonza Inc.) containing 100 mL trypsin/EDTA, 100 mL Trypsin Neutralizing Solution, and 100 mL HEPES Buffered Saline Solution. All the experiments were carried out with NHBE cultures between the PD 7 and 15 (maximum expected lifespan). Primary nasal epithelial cells were obtained from Laboratory donors by gently brushing the inferior turbinates using an endobrush (Aiesi Hospital Services, Naples, Italy). Cells were transported at room temperature in 2 mL of RPMI1640 medium (Lonza Inc.), complemented with 3% gentamicin. Cells were placed on an Eppendorf Thermomixer, in agitation at 300 rpm for 1 h. Once the brush was removed from each sample, cells were centrifuged at 931 g for 20', the supernatant was discarded, and cell pellet was treated with 150 mL of Trypsin-Versene EDTA solution (Lonza Inc.) for 4' at 37 °C, to disaggregate cell clusters. Trypsin was inactivated by adding 3 mL of serum-free bronchial epithelial cell growth medium BEGM (Lonza Inc.). After centrifugation at 2000 rpm for 10', cells were placed in CELL+ T 25 flasks (Sarstedt Ltd, Leicester, UK). Medium was changed daily. At confluence of 60%, cells were transferred in new flasks (about 3000 viable cells cm⁻² per flask). Cells were maintained in culture for 25 days, and a confluence of 60–80% was reached every 6–7 days. Each cell line was plated at different seeding densities and grown for the periods and under the conditions specified for each experiment. Cells were counted with an automated vision-based counter (Countess™ Automated Cell Counter; Gibco, Invitrogen). Population doubling was calculated at each passage until growth arrest by the following formula: PD = (log₁₀Y – log₁₀X)/log₂. Y indicates the number of cells counted at the end of the passage; X is the number of cells seeded. Cumulative population doubling was calculated as the sum of all the changes in PD.

Cellular senescence assay

Cells undergoing senescence typically express beta-galactosidase activity at pH 6. Beta-galactosidase-positive cells were detected with the Chemicon Cellular Senescence Assay kit (Millipore, Temecula, CA, USA). The day before the experiment, 300 000 cells were seeded in 35-mm petri dishes. They were washed twice with 2 mL 1X Ca/Mg-free phosphate-buffered saline (PBS) and fixed with 1 mL 1X fixing solution at room temperature for 15 min. After removing the fixing solution, the cells were washed twice with 2 mL 1X PBS and incubated overnight with 2 mL of freshly prepared 1X SA-beta-gal detection solution at 37 °C, without CO₂ and protected from light. Stained cells were washed twice with 2 mL 1X PBS to remove SA-beta-gal detection solution. Images were recorded using a light phase contrast microscope (Leica DM IL; Leitz, Wetzlar, Germany) linked to a Leica DFC320 camera.

Concanavalin A staining

IMR-90 cells, cultured on glass coverslips, were washed twice with cold PBS. Cells were then fixed with 4% paraformaldehyde-PBS buffer for 10 min and incubated with concanavalin A, Alexa FluorR 594 conjugate (Molecular Probes Inc., Invitrogen, Eugene, OR, USA) 20 mg mL⁻¹ for 20 min. After incubation, cells were washed 3–4 times with cold buffer and mounted, inverted, in ProLong Gold antifade reagent with DAPI (Molecular Probes Inc., Invitrogen), and observed with a fluorescence-inverted microscope (Leica Microsystems). Finally, fluorescence intensity was evaluated on the pictures with IMAGEJ (online free) software.

Assay for mannose-binding lectin binding to young and senescent cells

Lyophilized mannose-binding protein (MBP) was purchased from USBio-logical (Swampscott, MA, USA). Before use, we verified by electrophoresis that MBL correctly polymerized. Binding of human MBL protein to IMR-90, NHBE and primary nasal epithelial cells were evaluated using immunofluorescence. IMR-90 cells with different PDs (39, 46, and 54) were seeded on a 96-well clear bottom white polystyrene plate (Corning Incorporated, Corning, NY, USA) at 9×10^3 cells per well with 200 μL of medium 24 h before the experiment. The same procedure was done with NHBE cells (PD: 7, 10, and 15) and primary nasal epithelial cells (7, 14, and 25 days), but in these cases, the seeding density was 6×10^3 cells per well. The three cell lines were washed twice with 100 μL of 1X Hank's Balanced Salt Solution with Ca/Mg (Lonza Inc.) and were then treated with 50 μL of 5 $\mu\text{g mL}^{-1}$ human MBL for 1 h at room temperature. After incubation with MBP, cells were washed twice with 100 μL of 1X HBSS and then incubated with 50 μL of 4 $\mu\text{g mL}^{-1}$ anti-MBL mAb, D8.18 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 90 min at room temperature. Finally, cells were washed twice with 100 μL of 1X HBSS and incubated with 50 μL of 2 $\mu\text{g mL}^{-1}$ fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG for an additional 90 min at room temperature. Images of cells were acquired with a Leica DFC350FX monochrome digital camera connected to a Leica DMI4000B inverted fluorescence microscope.

Cytotoxicity assay

Cytotoxicity induced by MBL protein on senescent cells was assessed measuring LD in the culture medium using a commercial kit (Roche) with an automated analyzer.

Statistics

The chi square test with Yates correction was used to compare in the allelic frequency of each MBL2 haplotype and MBL2 genotypes in the three study groups. The unpaired *t*-test was used to compare serum albumin, MBL, and MBL/albumin ratio in the three study groups once verified that these parameters had a Gaussian distribution.

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Author contributions

All authors contributed equally to this work. R. Tomaiuolo administered the experiments and wrote the manuscript; C. Bellia, B. Lo Sasso, A. Ruocco, and C. Salapete designed and performed experiments; C. Franceschi and G. Baggio recorded clinical data from all subjects. C. Carru and A. Zinellu selected the population; M. Ciaccio, G. Castaldo, and L. Deiana designed experiments, analyzed data, and edited the paper. J. Vaupel and L. Deiana promoted the AKea study. All authors discussed the results and implications and commented on the manuscript at all stages.

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